

A VACCINE-INDUCED HEPATITIS B
VIRAL STRAIN AND USES THEREOF

Throughout this application, various references are referred
5 to within parentheses. Disclosures of these publications in
their entireties are hereby incorporated by reference into
this application to more fully describe the state of the art
to which this invention pertains.

10 BACKGROUND OF THE INVENTION

The present invention concerns the hepatitis B virus genome
with a vaccine-induced mutation at amino acid residue 145
(Glycine to Arginine) within the major surface antigen, its
15 nucleotide sequence, the deduced four major protein
sequences, antigen, antibody, detection systems, development
of effective vaccines, and antiviral agents.

The hepatitis B virus was first discovered in 1963 as a human
20 virus that is transmitted parenterally. Although these
viruses are not particularly cytotoxic and do not lead to
massive cell death, they have been the cause of a major
infectious disease affecting both adults and young children
worldwide. The presence of hepatitis B surface antigen has
25 served as the main detection marker for carriers of hepatitis
B virus, and thus, possibly those at risk of transmitting the
virus. Conversely, the occurrence of an anti-surface antigen
antibody indicates an immune response which would lead to
eventual recovery. Stimulation of such immune response has
30 been greatly helped by the currently licensed hepatitis B
vaccines developed by Merck Sharpe & Dohme. These vaccines
contain the major surface antigen in either the natural
(plasma-derived) or the recombinant (purified from yeast
cells) form, and have proven safe and effective in
35 neutralizing the hepatitis B virus. In Singapore, the active
vaccination program at a national scale has resulted in a
significant decrease of acute hepatitis B infection and the
incidence of primary hepatocellular carcinoma. This decrease
has in turn been associated with an increased immunity in the
40 population.

The major antigenic epitope of hepatitis B virus is a highly conserved region spanning 23 amino acid residues and located from amino acid position 124 to 147 of the major surface antigen. This small region designated as the group specific
5 determinant "a" is found in all subtypes and isolates of hepatitis B viral genomes. Its antigenic properties seem due to its proposed double loop structure, to which the vaccine-induced neutralizing antibody binds.

10 In contrast to random mutations introduced into hepatitis B viral genomes during viral replication by the proof-reading defective reverse transcriptase, mutations induced following vaccination occur mainly in the "a" epitope of the major surface antigen. These mutant viruses are of particular
15 interest since they show reduced affinity to the neutralizing antibody and therefore are able to replicate independently. Among these vaccine-escape mutants, the mutation at amino acid residue 145 (from Glycine to Arginine) in the second loop of the major surface antigen is the most significant
20 because it is stable, results in conformational changes of the "a" epitope and has been reported worldwide in North America, Europe, Japan and Southeast Asia. In Singapore, for example, such mutants are the most frequent variant following vaccination. Twelve infectious variants among 41
25 breakthroughs have been identified as having an arginine mutation at amino acid residue 145 of the major surface antigen. There is evidence of vertical transmission from one of the 12 variants and this variant has also been associated with active liver disease. Significantly, some of these
30 variants are now found in random asymptomatic adult population.

The occurrence of this replicative vaccine-induced mutant and its ability to escape detection using standard reagents is of
35 grave concern because it has resulted in the development of acute hepatitis B in Italy and Singapore. This situation therefore requires the urgent development of specific detection systems, as well as, effective prophylactic

vaccines and antiviral agents. Determination of the nucleotide sequence of this vaccine-induced mutant virus constitutes the first step towards these aims and will certainly be helpful for the various above-mentioned
s developments.

SUMMARY OF THE INVENTION

This invention provides an isolated strain of Hepatitis B virus designated Human Hepatitis B Virus Surface Antigen-'S'-145 Singapore Strain (Glycine to Arginine) which constituent viral genome is deposited under Accession Nos. P97121504, P97121505 and P97121506 with the European Collection of Cell Culture on 15th December 1997.

This invention also provides an isolated nucleic acid encoding a polypeptide which is a mutant major surface antigen of a strain of hepatitis B virus, such polypeptide having an amino acid sequence which differs from the amino acid sequence of a major surface antigen of a wild type hepatitis B virus in that the amino acid at position number 145 of such polypeptide is an arginine rather than a glycine.

This invention provides a method of producing the polypeptide in purified form and the resulting purified
20 polypeptide which is a mutant major surface antigen of a strain of hepatitis B virus, such polypeptide having an amino acid sequence which differs from the amino acid sequence of a major surface antigen of a wild type hepatitis B virus in that the amino acid at position number 145 of such
25 polypeptide is an arginine rather than a glycine.

This invention provides an oligonucleotide of at least 15 nucleotides capable of specifically hybridizing with sequences of only the mutant viral strain of hepatitis B
30 virus.

This invention provides a method of obtaining antibodies to a polypeptide which is a mutant major surface antigen of a strain of hepatitis B virus and the antibodies produced.
35

This invention provides uses of the above-described polypeptide, antibodies or nucleic acid for determining whether a subject is infected with the above-described viral

strain.

This invention also provides a composition capable of stimulating or enhancing antibody production for the
5 polypeptide.

This invention further provides a method for identifying a chemical compound which is capable of treating and/or preventing an infection by the above-described mutant viral
10 strain and compositions containing such compounds.

This invention also provides a composition comprising the chemical compound identified by the above-described methods in an amount effective to treat or prevent infection by the
15 strain and a pharmaceutically effective carrier.

This invention further provides use of compositions for treating a subject infected with this viral strain.

20 This invention also provides use of compositions for preventing infection of a subject by this viral strain.

And lastly, this invention provides a method of screening bodily fluids for this viral strain.

BRIEF DESCRIPTION OF THE FIGURES

- Figure 1. Structure of the four open reading frames of human hepatitis B viral genome isolated from a male, eleven year old Singaporean child with a glycine to arginine mutation at amino acid residue 145 of the major surface antigen, as labeled by an asterisk. The major viral proteins: DNA polymerase, large/middle/major surface antigen, precore, core and transactivating X are denoted as P, Pres 1/Pres2/S, PreC, C and X respectively.
- Figure 2. Strategy of cloning and sequence determination of the same hepatitis B viral genome.
- Figure 3. Whole nucleotide sequence of human hepatitis B virus, isolated from an eleven year old child born to a mother in Singapore with the wild type virus. The child had received standard Hepatitis B immunoglobulin (HBIG) and HB vaccine and was infected with the mutated strain one year later. This strain carries a mutation at amino acid residue 145 (glycine to arginine) of the major surface antigen (SEQ. I.D. No. 1). The mutation is shown at nucleic acids numbered 587-589.
- Figure 4. Deduced amino acid sequence of the DNA polymerase from the nucleotide sequence of Figure 3 (SEQ. I.D. No. 2).
- Figure 5. Deduced amino acid sequence of the large surface antigen from the nucleotide sequence of Figure 3. The mutated amino acid residue (G to R) is numbered 319 (SEQ. I.D. No. 3).

- Figure 6. Deduced amino acid sequence of the core protein from the nucleotide sequence of Figure 3 (SEQ. I.D. No. 4).
- 5 Figure 7. Deduced amino acid sequence of the trans-activating X protein from the nucleotide sequence of Figure 3 (SEQ. I.D. No. 5).
- 10 Figure 8. Oligonucleotide sequence corresponding to the initiation site of the coding region of DNA polymerase, at position 2307 of the viral genome and matches the coding strand (sense oligonucleotide) (SEQ.I.D.No.6).
- 15 Figure 9. Oligonucleotide sequence corresponding to position 250 of the viral nucleotide sequence and matches the complementary strand (anti-sense oligonucleotide) (SEQ.I.D.No.7).
- 20 Figure 10. Oligonucleotide sequence corresponding to position 250 of the viral nucleotide sequence and matches the coding strand (sense oligonucleotide) (SEQ.I.D.No.8).
- 25 Figure 11. Oligonucleotide sequence corresponding to the stop codon of the coding region of DNA polymerase, at position 1623 of the viral genome and matches the complementary strand (anti-sense oligonucleotide) (SEQ.I.D.No.9).
- 30 Figure 12. Oligonucleotide sequence corresponding to position 1420 of the viral genome and matches the coding strand (sense oligonucleotide) (SEQ.I.D.No.10).
- 35 Figure 13. Oligonucleotide sequence corresponding to position 2340 of the viral genome and matches

8

the complementary strand (anti-sense
oligonucleotide) (SEQ.I.D.No.11).

DETAILED DESCRIPTION OF THE INVENTION

Throughout this application, references to specific nucleotides are to nucleotides present on the coding strand of the nucleic acid. The following standard abbreviations are used throughout the specification to indicate specific nucleotides:

	C=cytosine	A=adenosine
10	T=thymidine	G=guanosine

The present invention provides the nucleotide sequence of a hepatitis B virus genome, which carries a vaccine-induced mutation at amino acid residue 145 (Glycine to Arginine) of the major surface antigen, consisting of 3215 nucleotides (Figure 3) coding for 4 overlapping viral proteins shown in Figures 4-7.

The invention provides amino acid sequences of the four major viral proteins, these include the DNA polymerase, large/middle/major surface antigen, core and trans-activating X. These proteins can be produced using recombinant technology, and used in developing polyclonal or monoclonal antibodies.

The present invention also provides a hepatitis B virus diagnostic system, specific for the vaccine-induced mutation at amino acid residue 145 (Glycine to Arginine) of the major surface antigen, using nucleotide or protein sequences or antibodies described herein.

The present invention provides an isolated strain of Hepatitis B virus designated Human Hepatitis B Virus Surface Antigen-'S'-145 Singapore Strain (Glycine to Arginine) which constituent viral genome is deposited under Accession Nos. P97121504, P97121505 and P97121506.

The invention also provides an isolated nucleic acid encoding

a polypeptide which is a mutant major surface antigen of a strain of hepatitis B virus, such polypeptide having an amino acid sequence which differs from the amino acid sequence of the major surface antigen of a wild type hepatitis B virus in that the amino acid at position number 145 of such polypeptide is an arginine rather than a glycine. In a specific embodiment, the polypeptide is being encoded by nucleotides 155 through 835 of the nucleic acid sequence designated SEQ. I.D. No. 1, specifically, comprising nucleotides "AGA" in position 587-589, instead of "GGA." This nucleic acid can be DNA or RNA, specifically, cDNA or genomic DNA.

In another embodiment of the invention, the polypeptide has an amino acid sequence substantially the same as amino acid residues 174 through 400 of the amino acid sequence designated SEQ. I.D. No. 3.

This invention further provides an isolated nucleic acid which encodes a peptide, wherein the peptide is encoded by a nucleic acid molecule comprising nucleotides 527 through 595 of SEQ. I.D. No. 1.

This invention also provides an isolated nucleic acid which encodes a peptide, wherein the peptide has an amino acid sequence comprising amino acid residues 298 through 320 of the amino acid sequence designated SEQ. I.D. No. 3.

This invention also provides a vector comprising an isolated nucleic acid encoding a polypeptide which is a mutant major surface antigen of a strain of hepatitis B virus, such polypeptide having an amino acid sequence which differs from the amino acid sequence of a major surface antigen of a wild type hepatitis B virus in that the amino acid at position number 145 of such polypeptide is an arginine rather than a glycine and operatively linked to a promoter of RNA transcription.

Further, this invention provides a vector comprising an isolated nucleic acid encoding a peptide, wherein the peptide is encoded by a nucleic acid molecule comprising nucleotides 527 through 595 of SEQ. I.D. No. 1.

In both of the above-identified vectors, the vector may comprise viral DNA.

This invention also provides a host vector system for the production of a polypeptide which comprises the above-described vectors in a suitable host.

This invention also provides a method of producing a polypeptide or a peptide which comprises growing the host vector systems described above, under suitable conditions permitting production of the polypeptide and recovering the polypeptide so produced.

This invention further provides a method of obtaining a polypeptide or a peptide in purified form which comprises:
20 (a) introducing the above-described vectors into a suitable host cell; (b) culturing the resulting host cell so as to produce the polypeptide; (c) recovering the polypeptide produced in step (b); and (d) purifying the polypeptide so
25 recovered.

This invention further provides a purified polypeptide which is a mutant major surface antigen of a strain of hepatitis B virus, such polypeptide having an amino acid sequence which
30 differs from the amino acid sequence of a major surface antigen of a wild type hepatitis B virus in that the amino acid at position number 145 of such polypeptide is an arginine rather than a glycine. One means of obtaining the polypeptide is by the above-described method.

35

This invention also provides a purified peptide, wherein the peptide has an amino acid sequence comprising amino acid residues 298 through 320 of the amino acid sequence

designated Seq. I.D. No. 3. One means of obtaining this peptide is by the above-described method.

This invention also provides an oligonucleotide of at least
5 15 nucleotides capable of specifically hybridizing with a
unique sequence of nucleotides within a nucleic acid which
encodes a polypeptide which is a mutant major surface antigen
of a strain of hepatitis B virus, such polypeptide having an
amino acid sequence which differs from the amino acid
10 sequence of a major surface antigen of a wild type hepatitis
B virus in that the amino acid at position number 145 of such
polypeptide is an arginine rather than a glycine, without
hybridizing to any sequence of nucleotides within a nucleic
acid which encodes the major surface antigen of a wild type
15 hepatitis B virus. Specifically the oligonucleotide comprises
nucleotides 527 through 595 of SEQ. I.D. No. 1.

This invention also provides a method of obtaining antibodies
to a polypeptide which is a mutant major surface antigen of
20 a strain of hepatitis B virus, such polypeptide having an
amino acid sequence which differs from the amino acid
sequence of a major surface antigen of a wild type hepatitis
B virus in that the amino acid at position number 145 of such
polypeptide is an arginine, rather than a glycine, and not to
25 the major surface antigen of a wild type hepatitis B virus,
comprising: (a) obtaining the polypeptide in a purified form;
(b) immunizing an organism capable of producing antibodies
against the purified polypeptide; (c) collecting the produced
antibodies; (d) combining the produced antibodies and the
30 purified polypeptide under conditions to form a complex; and
(e) determining which produced antibodies form a complex with
the purified polypeptide so as to obtain antibodies to the
polypeptide. Specifically, the polypeptide is being encoded
by nucleotides 155 through 835 of the nucleic acid sequence
35 designated SEQ. I.D. No. 1. In another embodiment, the
polypeptide has an amino acid sequence substantially
identical to amino acid residues 174 through 400 of the amino
acid sequence designated SEQ. I.D. No. 3.

One could perform the above-described well-known method in rabbits or mice.

This invention also provides a method of obtaining antibodies to a peptide, wherein the peptide has an amino acid sequence comprising amino acid residues 298 through 320 of the amino acid sequence designated SEQ. I.D. No. 3, comprising: (a) obtaining the peptide in a purified form; (b) immunizing an organism capable of producing antibodies against the purified peptide; (c) collecting the produced antibodies; (d) combining the produced antibodies and the purified peptide under conditions to form a complex; and (e) determining which produced antibodies form a complex with the purified peptide so as to obtain antibodies to the peptide.

One could perform the above-described well-known method in rabbits or mice.

This invention also provides the antibodies obtained from the above-described methods, specifically the monoclonal antibodies. Further, the invention provides antibodies capable of detecting a polypeptide which is a mutant major surface antigen of a strain of hepatitis B virus, such polypeptide having an amino acid sequence which differs from the amino acid sequence of a major surface antigen of a wild type hepatitis B virus in that the amino acid at position number 145 of such polypeptide is an arginine, rather than a glycine, and incapable of detecting the major surface antigen of a wild type hepatitis B virus, as well as, antibodies capable of detecting a peptide, wherein the peptide has an amino acid sequence comprising amino acid residues 298 through 320 of the amino acid sequence designated SEQ. I.D. No. 3.

This invention also provides use of an isolated nucleic acid encoding a polypeptide which is a mutant major surface antigen of a strain of hepatitis B virus, such polypeptide having an amino acid sequence which differs from the amino

acid sequence of a major surface antigen of a wild type hepatitis B virus in that the amino acid at position number 145 of such polypeptide is an arginine, rather than a glycine for determining whether a subject is infected with a strain
5 of Hepatitis B virus designated Human Hepatitis B Virus Surface Antigen-'S'-145 Singapore Strain (Glycine to Arginine), wherein such determination comprises: (a) obtaining an appropriate nucleic acid sample from the subject; and (b) determining whether the nucleic acid sample
10 from step (a) is, or is derived from, a nucleic acid encoding a polypeptide which is a mutant major surface antigen of a strain of hepatitis B virus, such polypeptide having an amino acid sequence which differs from the amino acid sequence of a major surface antigen of a wild type hepatitis B virus in
15 that the amino acid at position number 145 of such polypeptide is an arginine, rather than a glycine.

A means of determination is where the nucleic acid sample in step (a) comprises mRNA corresponding to the transcript of
20 DNA encoding a polypeptide which is a mutant major surface antigen of a strain of hepatitis B virus, such polypeptide having an amino acid sequence which differs from the amino acid sequence of a major surface antigen of a wild type hepatitis B virus in that the amino acid at position number
25 145 of such polypeptide is an arginine, rather than a glycine, and wherein the determining of step (b) comprises: (i) contacting the mRNA with the above-described oligonucleotide under conditions permitting binding of the mRNA to the oligonucleotide so as to form a complex; (ii)
30 isolating the complex so formed; and (iii) identifying the mRNA in the isolated complex so as to thereby determine whether the mRNA is, or is derived from, a nucleic acid which encodes the polypeptide.

35 Another example is where the nucleic acid sample in step (a) comprises mRNA corresponding to the transcript of DNA encoding a polypeptide which is a mutant major surface antigen of a strain of hepatitis B virus, such polypeptide

having an amino acid sequence which differs from the amino acid sequence of a major surface antigen of a wild type hepatitis B virus in that the amino acid at position number 145 of such polypeptide is an arginine, rather than a glycine, and wherein the determining of step (b) comprises: (i) translating the mRNA under suitable conditions to obtain an amino acid sequence; and (ii) comparing the amino acid sequence of step (i) with the amino acid sequence of an isolated nucleic acid which encodes a polypeptide, wherein the polypeptide has an amino acid sequence substantially identical to amino acid residues 174 through 400 of the amino acid sequence designated SEQ. I.D. No. 3 so as to thereby determine whether the nucleic acid sample is, or is derived from, a nucleic acid which encodes the polypeptide. A further example is where the determining of step (b) comprises: (i) amplifying the nucleic acid present in the sample of step (a); and (ii) detecting the presence of polypeptide in the resulting amplified nucleic acid.

This invention provides the use of an antibody that recognizes a polypeptide which is a mutant major surface antigen of a strain of hepatitis B virus for determining whether the subject has a predisposition for hepatocellular carcinoma, wherein such determination comprises: (a) obtaining an appropriate nucleic acid sample from the subject; and (b) determining whether the nucleic acid sample from step (a) is, or is derived from, a nucleic acid encoding a polypeptide which is a mutant major surface antigen of a strain of hepatitis B virus, such polypeptide having an amino acid sequence which differs from the amino acid sequence of a major surface antigen of a wild type hepatitis B virus in that the amino acid at position number 145 of such polypeptide is an arginine, rather than a glycine, by contacting the sample under appropriate conditions to bind to the antibodies of claim 35 so as to determine whether the subject has a predisposition for hepatocellular carcinoma.

This invention also provides use of antibodies capable of

detecting a polypeptide which is a mutant major surface antigen of a strain of Hepatitis B virus designated Human Hepatitis B Virus Surface Antigen-'S'-145 Singapore Strain (Glycine to Arginine) for determining whether a subject is
5 infected with a strain of Hepatitis B virus designated Human Hepatitis B Virus Surface Antigen-'S'-145 Singapore Strain (Glycine to Arginine), wherein such determination comprises: (a) obtaining an appropriate sample from the subject; and (b) determining whether the sample from step (a) is, or is derived
10 from, a nucleic acid encoding a polypeptide which is a mutant major surface antigen of a strain of hepatitis B virus, such polypeptide having an amino acid sequence which differs from the amino acid sequence of a major surface antigen of a wild type hepatitis B virus in that the amino acid at position
15 number 145 of such polypeptide is an arginine, rather than a glycine by contacting the sample under appropriate conditions to bind to the antibodies so as to determine whether a subject is infected. Furthermore, the antibody may also be capable detecting a peptide, wherein the peptide has an amino acid
20 sequence comprising amino acid residues 298-320 of the amino acid sequence designated SEQ. I.D. No. 3.

In the above-described uses, the isolated nucleic acid, oligonucleotide or antibody may be labeled with a detectable
25 marker. Examples of detectable markers include radioactive isotopes, fluorophors and enzymes.

In a specific embodiment, the sample includes, but is not limited to, blood, tissue or sera.

30 This invention also provides a method for identifying a chemical compound for the manufacture of a medicament which is capable of treating infection by a strain of Hepatitis B virus designated Human Hepatitis B Virus Surface Antigen-'S'-145
35 Singapore Strain (Glycine to Arginine) which comprises: (a) contacting a polypeptide which is a mutant major surface antigen of a strain of hepatitis B virus, such polypeptide having an amino acid sequence which differs from the amino

acid sequence of a major surface antigen of a wild type hepatitis B virus in that the amino acid at position number 145 of such polypeptide is an arginine, rather than a glycine, with the chemical compound under conditions permitting binding
5 between the polypeptide and the chemical compound; (b) detecting specific binding of the chemical compound to the polypeptide; and (c) determining whether the chemical compound inhibits the polypeptide so as to identify a chemical compound which is capable of treating infection by the viral strain.

10

This invention also provides a method for identifying a chemical compound for the manufacture of a medicament which is capable of preventing infection by a strain of Hepatitis B virus designated Human Hepatitis B Virus Surface Antigen-'S'-
15 145 Singapore Strain (Glycine to Arginine), which comprises: (a) contacting a polypeptide which is a mutant major surface antigen of a strain of hepatitis B virus, such polypeptide having an amino acid sequence which differs from the amino acid sequence of a major surface antigen of a wild type
20 hepatitis B virus in that the amino acid at position number 145 of such polypeptide is an arginine, rather than a glycine, with the chemical compound under conditions permitting binding between the polypeptide and the chemical compound; (b) detecting specific binding of the chemical compound to the
25 polypeptide; and (c) determining whether the chemical compound inhibits the polypeptide so as to identify a chemical compound which is capable of preventing infection by the viral strain.

This invention further provides a composition comprising a
30 polypeptide which is a mutant major surface antigen of a strain of hepatitis B virus, such polypeptide having an amino acid sequence which differs from the amino acid sequence of a major surface antigen of a wild type hepatitis B virus in that the amino acid at position number 145 of such polypeptide is
35 an arginine, rather than a glycine, or derivative thereof, the amounts of such polypeptide being effective to stimulate or enhance antibody production in a subject, and a pharmaceutically acceptable carrier.

The actual effective amount will be based upon the size of the polypeptide, the biodegradability of the polypeptide, the bioactivity of the polypeptide and the bioavailability of the polypeptide. If the polypeptide does not degrade quickly, is bioavailable and highly active, a smaller amount will be required to be effective. The effective amount will be known to one of skill in the art; it will also be dependent upon the form of the polypeptide, the size of the polypeptide and the bioactivity of the polypeptide. Use of an adjuvant for example, would lower the required amount of the polypeptide. One of skill in the art could routinely perform empirical activity tests to determine the bioactivity in bioassays and thus determine the effective amount.

Pharmaceutically acceptable carriers are well known to those skilled in the art and include, but are not limited to, 0.01-0.1M and preferably 0.05M phosphate buffer or 0.8% saline. Additionally, such pharmaceutically acceptable carriers may be aqueous or non-aqueous solutions, suspensions, and emulsions.

20 Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media.

25 Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers such as those based on Ringer's dextrose, and the like. Preservatives and other

30 additives may also be present, such as, for example, antimicrobials, antioxidants, chelating agents, inert gases and the like.

This invention further provides a composition comprising a

35 peptide, wherein the peptide has an amino acid sequence comprising amino acid residues 298 through 320 of the amino acid sequence designated SEQ. I.D. No. 3 or derivative thereof, the amounts of such peptide being effective to

stimulate or enhance antibody production in a subject, and a pharmaceutically acceptable carrier.

5 This invention further provides compositions comprising the chemical compound identified by the above-described methods in an amount effective to treat or prevent infection by a strain of Hepatitis B virus designated Human Hepatitis B Virus Surface Antigen-'S'-145 Singapore Strain (Glycine to Arginine) and a pharmaceutically effective carrier.

10 This invention provides the use of the above-described compositions as medicaments for treating and/or preventing hepatocellular carcinoma.

15 This invention also provides use of the above-identified compositions for treating a subject infected with a strain of Hepatitis B virus designated Human Hepatitis B Virus Surface Antigen-'S'-145 Singapore Strain (Glycine to Arginine).

20 This invention also provides use of the above-identified compositions for preventing infection by a strain of Hepatitis B virus designated Human Hepatitis B Virus Surface Antigen-'S'-145 Singapore Strain (Glycine to Arginine) in a subject which comprises administering an effective amount.

25 This invention further provides a method of screening tissues and bodily fluids from a subject for a strain of hepatitis B virus designated Human Hepatitis B Virus Surface Antigen-'S'-145 Singapore Strain (Glycine to Arginine) which comprises:
30 (a) obtaining an appropriate sample of bodily fluid from the subject; (b) determining the presence of a polypeptide which is a mutant major surface antigen of a strain of hepatitis B virus, such polypeptide having an amino acid sequence which differs from the amino acid sequence of a major surface
35 antigen of a wild type hepatitis B virus in that the amino acid at position number 145 of such polypeptide is an arginine, rather than a glycine in the sample of step (a) so as to screen the sample for the strain. In embodiments of

this method, the bodily fluid comprises blood, sera, or a nucleic acid sample of blood or sera.

This invention provides a method for determining whether a
5 subject has a predisposition for hepatocellular carcinoma,
which comprises: (a) obtaining an appropriate nucleic acid
sample from the subject; and (b) determining whether the
nucleic acid sample from step (a) is, or is derived from, a
nucleic acid encoding a polypeptide which is a mutant major
10 surface antigen of a strain of hepatitis B virus, such
polypeptide having an amino acid sequence which differs from
the amino acid sequence of a major surface antigen of a wild
type hepatitis B virus in that the amino acid at position
number 145 of such polypeptide is an arginine, rather than a
15 glycine, thereby determining whether the subject has a
predisposition for hepatocellular carcinoma.

This invention also provides the above-described method,
wherein the nucleic acid sample in step (a) comprises mRNA
20 encoding a polypeptide which is a mutant major surface antigen
of a strain of hepatitis B virus, such polypeptide having an
amino acid sequence which differs from the amino acid sequence
of a major surface antigen of a wild type hepatitis B virus in
that the amino acid at position number 145 of such polypeptide
25 is an arginine, rather than a glycine, and wherein the
determining of step (b) comprises: (i) contacting the mRNA
with the above-described oligonucleotides under conditions
permitting binding of the mRNA to the oligonucleotide so as to
form a complex; (ii) isolating the complex so formed; and
30 (iii) identifying the mRNA in the isolated complex so as to
thereby determine whether the mRNA is, or is derived from, a
nucleic acid which encodes the polypeptide.

This invention further provides the above-described method,
35 wherein the nucleic acid sample in step (a) comprises mRNA
encoding a polypeptide which is a mutant major surface antigen
of a strain of hepatitis B virus, such polypeptide having an
amino acid sequence which differs from the amino acid sequence

of a major surface antigen of a wild type hepatitis B virus in that the amino acid at position number 145 of such polypeptide is an arginine, rather than a glycine, and wherein the determining of step (b) comprises: (i) translating the mRNA
5 under suitable conditions to obtain an amino acid sequence; and (ii) comparing the amino acid sequence of step (i) with the amino acid sequence encoded by the isolated nucleic acid described above so as to determine whether the nucleic acid sample is, or is derived from, a nucleic acid which encodes
10 the polypeptide.

This invention also provides the above-described method, wherein the determining of step (b) comprises: (i) amplifying the nucleic acid present in the sample of step (a); and (ii)
15 detecting the presence of polypeptide in the resulting amplified nucleic acid.

This invention further provides the above-described method for determining whether a subject has a predisposition for
20 hepatocellular carcinoma, which comprises: (a) obtaining an appropriate sample from the subject; and (b) determining whether the sample from step (a) is, or is derived from, a nucleic acid encoding a polypeptide which is a mutant major surface antigen of a strain of hepatitis B virus, such
25 polypeptide having an amino acid sequence which differs from the amino acid sequence of a major surface antigen of a wild type hepatitis B virus in that the amino acid at position number 145 of such polypeptide is an arginine, rather than a glycine, by contacting the sample under appropriate conditions
30 to bind to the above-described antibodies so as to determine whether the subject has a predisposition for hepatocellular carcinoma.

This invention provides the above-described methods, wherein
35 the oligonucleotide or antibody is labeled with a detectable marker.

This invention also provides the above-described methods,

wherein the detectable marker is a radioactive isotope, a fluorophor or an enzyme.

This invention also provides the above-described methods,
5 wherein the sample comprises blood, tissue or sera.

This invention further provides a method for identifying a chemical compound for the manufacture of a medicament which is capable of treating hepatocellular carcinoma which comprises:
10 (a) contacting a polypeptide which is a mutant major surface antigen of a strain of hepatitis B virus, such polypeptide having an amino acid sequence which differs from the amino acid sequence of a major surface antigen of a wild type hepatitis B virus in that the amino acid at position number
15 145 of such polypeptide is an arginine, rather than a glycine, with the chemical compound under conditions permitting binding between the polypeptide and the chemical compound; (b) detecting specific binding of the chemical compound to the polypeptide; and (c) determining whether the chemical compound
20 binds to the polypeptide so as to identify a chemical compound which is capable of treating hepatocellular carcinoma.

This invention provides a method for identifying a chemical compound for the manufacture of a medicament which is capable
25 of preventing hepatocellular carcinoma, which comprises: (a) contacting a polypeptide which is a mutant major surface antigen of a strain of hepatitis B virus, such polypeptide having an amino acid sequence which differs from the amino acid sequence of a major surface antigen of a wild type
30 hepatitis B virus in that the amino acid at position number 145 of such polypeptide is an arginine, rather than a glycine, with the chemical compound under conditions permitting binding between the polypeptide and the chemical compound; (b)
35 detecting specific binding of the chemical compound to the polypeptide; and (c) determining whether the chemical compound binds to the polypeptide so as to identify a chemical compound which is capable of preventing hepatocellular carcinoma.

Additionally, this invention provides a composition comprising the chemical compound identified by the above-described methods in an amount effective to treat hepatocellular carcinoma and a pharmaceutically effective carrier.

5

This invention also provides a composition comprising the chemical compound identified by the above-described methods in an amount effective to prevent hepatocellular carcinoma and a pharmaceutically effective carrier.

10

This invention further provides a method treating a subject with hepatocellular carcinoma which comprises administering an effective amount of the above-described compositions.

15

This invention further provides a method preventing hepatocellular carcinoma in a subject which comprises administering an effective amount of the above-described compositions.

20

This invention also provides a hepatitis vaccine, comprising a mutant form of the surface antigen of hepatitis B virus, such polypeptide having an amino acid sequence which differs from the amino acid sequence of the major surface antigen of hepatitis B in that the amino acid at position number 145 of

25

such polypeptide is an arginine rather than a glycine.

This invention also provides the above-described vaccine and an adjuvant.

30

This invention is illustrated in the Experimental Details section which follows. These sections are set forth to aid in an understanding of the invention but are not intended to, and should not be construed to, limit in any way the invention as set forth in the claims which follow thereafter.

35

Experimental Details

In the method described below, the hepatitis B virus carrying

the mutation at amino acid residue 145 of the major surface antigen was isolated, and its nucleotide sequence was determined.

5 Serum sample (20/815c) was obtained from an 11-year old child born to a mother carrying the wild type surface antigen. The child tested surface antigen negative at birth and was subjected to a combined treatment of hepatitis B immunoglobulin (HBIG) and plasma-derived vaccine. He was
10 tested surface antigen positive one year later and carried an Arginine mutation at amino acid residue 145 in the major surface antigen. The viral DNA was extracted prior to the determination of its sequence in the present invention.

15 As described in the Examples below, the genome of this hepatitis B mutant virus carrying a mutation at amino acid residue 145 of the major surface antigen consists of 3215 nucleotides which are identical to those of the wild type virus of the same subtype (adw). Open reading frames (ORFs)
20 coding for the major viral proteins are found at corresponding positions when compared to the wild type virus. Position 1 in the mutant hepatitis B virus genome is defined according to that in the wild type virus, corresponding to the restriction site EcoRI which is absent in the hepatitis B virus in the
25 present invention due to changes in the nucleotide sequence.

The structure of the different ORFs in the mutant virus genome reported are summarized in Figure 1 and their locations are indicated as follows:

- 30 - DNA polymerase gene starts at position 2307 and ends at position 1623, therefore consisting of 2532 nucleotides and coding for 843 amino acid residues;
- Large surface antigen gene starts at position 2848 and ends at position 835, therefore consisting of 1203 nucleotides and
35 coding for 400 amino acid residues. This large surface antigen overlaps the middle surface antigen starting at position 3205 and the major surface antigen which starts at position 155. Both the middle (consisting of 281 amino acids

residues) and the major (consisting of 226 amino acid residues) surface antigens end at the same position as the large surface antigen;

- Core gene starts at position 1814 and ends at position 2452, therefore consisting of 639 nucleotides and coding for 212 amino acid residues; and
- Trans-activating X gene starts at position 1374 and ends at position 1838, therefore consisting of 465 nucleotides and coding for 154 amino acid residues.

Furthermore, sequence analysis has established this mutant hepatitis B virus as belonging to adw subtype, as indicated by lysine residue at both positions 122 and 160 in the major surface antigen. Consistent with previous analysis of the "a" subtype by direct sequencing, the vaccine-induced mutation (from Glycine to Arginine) is found at amino acid residue 145 of the major surface antigen.

20 Compared with the wild type hepatitis B virus deposited in the Genbank database (accession number D00329), the identity of this hepatitis B viral strain is at 89.4% for the nucleotide sequence. The identity of different viral proteins of the present mutant hepatitis B virus as compared with its wild type counterparts is at 88.3%, 87.7%, 93.4% and 87% for DNA
25 polymerase (PIR - Protein Identification Resources accession number P93460), large surface antigen (PIR accession number A93460), core (PIR accession number C93460) and trans-activating X (PIR accession number A31289) proteins, respectively.

30

The hepatitis B virus genome in the present invention carrying a vaccine-induced mutation at amino acid residue 145 (Glycine to Arginine) of the major surface antigen, can be used as material to design oligonucleotides specific to the mutant
35 virus genome. These oligonucleotides can be used as material for highly specific diagnostic agents that detect virus carrying a vaccine-induced mutation at amino acid residue 145 of the major surface antigen.

The hepatitis B virus genome in the present invention, with a vaccine-induced mutation at amino acid residue 145 (Glycine to Arginine) of the major surface antigen, can be used as material to produce proteins of the invention by expressing a
5 vector that carries the relevant coding region, and which can replicate in a host cell such as Escherichia coli, by standard DNA recombinant technology.

Proteins of the present invention are useful as material for
10 highly specific diagnostic agents capable of detecting hepatitis B virus carrying a vaccine-induced mutation at amino acid residue 145 of the major surface antigen. Using known methods, these same proteins can be used to produce polyclonal and monoclonal antibodies.

15 Polyclonal and monoclonal antibodies can be used as material for diagnostic agents to detect with high specificity antigens of hepatitis B virus, with a vaccine-induced mutation at amino acid residue 145 (Glycine to Arginine) of the major surface
20 antigen.

A detection system using each protein of the present invention or proteins with partial replacement of amino acids, and a detection system using monoclonal or polyclonal antibodies to
25 such proteins, are useful as highly specific diagnostic agents for hepatitis B virus with vaccine-induced mutation at amino acid residue 145 of the major surface antigen, and are effective to detect and screen out such virus from transfusion bloods or blood derivatives. The proteins or antibodies to
30 such proteins, can be used as a material for development of prophylactic and therapeutic vaccine against such virus.

It is well known that one or more nucleotides in a DNA sequence can be substituted by other nucleotides to produce
35 the same protein. The present invention also concerns such nucleotide changes which code for proteins reported in this invention. It is equally well known that one or more amino acids in a protein sequence can be replaced by other analogous

amino acids, as defined by their hydrophilic properties or charges, to produce an analog of the amino acid sequence. Any analogs of the proteins of the present invention involving amino acid replacement, deletions, or by isosteres (modified amino acids that bear close structural and spatial similarity to protein amino acids), amino acid addition, or isostere addition can be utilized, provided that the resulting sequences elicit antibodies recognizing hepatitis B virus with a vaccine-induced mutation at amino acid mutation 145 (Glycine to Arginine) of the major surface antigen.

EXAMPLES

The nucleotide sequence and the deduced amino acid sequence of hepatitis B virus, carrying a vaccine-induced mutation at amino acid residue 145 (Glycine to Arginine) of the major surface antigen, were determined in the following manner:

1. Isolation of Viral DNA

The viral DNA was isolated from a serum sample (20/815c) obtained from the serum of an 11-year old Chinese child who was born to a mother of wild type hepatitis B virus surface antigen. Despite negative test results at birth, he was given combined treatment of HBIG and the plasma-derived vaccine and tested positive for hepatitis B virus surface antigen one year later. Sequence analysis of the "a" epitope indicated the presence of a vaccine-induced mutation at amino acid residue 145 of the major surface antigen.

The isolation method used was:

200 μ l of the serum sample was added with 400 μ l of the lysis buffer (Tris chloride 10 mM, pH7.4, EDTA 1 mM, and sodium dodecyl sulfate 2%) and 25 μ l of proteinase K (20 mg/ml), incubated at 65 C for 3 hours. Viral DNA was then extracted by phenol/chloroform and precipitated by ethanol.

2. Amplification of Viral DNA by Polymerase Chain Reaction

(PCR)

- The virus genome was amplified by polymerase chain reaction (PCR) using 3 sets of overlapping oligonucleotides, which were designed according to the wild type hepatitis B virus of adw subtype. Various restriction enzyme sites were included to facilitate the cloning of the PCR products. The position of these oligonucleotides is shown in Fig. 2 and indicated as follows:
- 10 - Flag 1 (ATAAGCTTATGCCCCTATCTTATCAACACTTCCGGA) (SEQ. I.D. No. 6) starts at the initiation site of the coding region of DNA polymerase, at position 2307 of the viral nucleotide sequence and matches the coding strand (sense oligonucleotide). An additional HindIII restriction enzyme site is underlined;
 - 15 - Xba3 (GAGTCTAGACTCTGCGGTATTGTGA) (SEQ. I.D. No. 7) starts at the internal restriction enzyme site XbaI, at position 250 of the viral nucleotide sequence and matches the complementary strand (anti-sense oligonucleotide). An additional XbaI restriction enzyme site is underlined;
 - 20 - Xba5 (GAGTCTAGACTCGTGGTGGACTTCT) (SEQ. I.D. No. 8) starts at the internal XbaI site, at the same location as that of Xba3 oligonucleotide but matches the coding strand (sense oligonucleotide). An additional XbaI restriction enzyme site is underlined;
 - 25 - Common 3 (TGAGAATTCTCACGGTGGTCTCCATGCGACGT) (SEQ. I.D. No. 9) starts at the stop codon of the DNA polymerase, at position 1623 of the viral nucleotide sequence and matches the complementary strand (anti-sense oligonucleotide). An additional EcoRI restriction enzyme site is underlined;
 - 30 - V11 (TTTGTTTACGTCCCGT) (SEQ. I.D. No. 10) starts near the initiation site of the X gene, at position 1420 of the viral nucleotide sequence and matches the coding strand (sense oligonucleotide);
 - HindIIIADW3 (CTAAGCTTAGTTTCCGGAAGTGTGAT) (SEQ. I.D. No. 11)
 - 35 starts close to the initiation site of the DNA polymerase, at position 2340 and matches the complementary strand (anti-sense oligonucleotide). An additional Hind III restriction enzyme site is underlined.

Using viral DNA as a template, PCR was then carried out on a DNA Thermal Cycler (Perkin-Elmer, Cetus) for 35 cycles using Pfu polymerase (Stratagene, U.S.A.), each cycle consisting of 1.5 minutes at a denaturing temperature of 94 °C, 2 minutes at an annealing temperature of 53 °C and 4 minutes at an extension temperature of 72 °C. The following combinations of oligonucleotides were used: Flag1/Xba3, Xba5/Common3 and V11/HindIIIADW3, and generating amplification products of 1.2 kb, 1.4 kb and 1.1 kb, respectively.

10

3. Cloning of the Amplified Viral DNA Fragments.

Amplified viral DNA fragment from Flag1/Xba3 (1.2 kb) was subjected to restriction enzyme digestion by HindIII and XbaI prior to cloning in a BlueScript plasmid pre-treated by the same restriction enzymes. Similar digestion with XbaI and EcoRI was applied to PCR product from Xba5/Common3 (1.4 kb) prior to cloning in a BlueScript plasmid pre-treated by XbaI and EcoRI. On the other hand, the DNA fragment amplified with V11 and HindIIIADW3 (1.1 kb) was directly cloned into ZeroBlunt plasmid, developed by InvitroGen (U.S.A.) for cloning blunt-end DNA fragments.

20

4. Determination of Nucleotide Sequence

25

Nucleotide sequence of the vaccine-induced hepatitis B virus in the present invention was determined on plasmid DNA template by chain-terminating inhibitors, using the Sequenase DNA Sequencing Kit (United States Biochemical Corp.). To facilitate the sequencing procedure, various internal oligonucleotides were designed (from V1 to V13) according to the wild type hepatitis B virus, and their positions are indicated in Fig. 2.

30

From the analysis described above, the full-length nucleotide sequence of the hepatitis B virus carrying a vaccine-induced mutation at amino acid residue 145 (Glycine to Arginine) of the major surface antigen was determined as shown in Figure 3.

35

The deduced amino acid sequences coding for the major viral proteins are shown in Figures 4-7: hepatitis B viral DNA polymerase (Figure 4), the large surface antigen (Figure 5), the core protein (Figure 6) and the trans-activating X protein
5 (Figure 7).

Alignment of the virus sequence in the present invention with other hepatitis B viral sequences, available in the Genbank database, will point to specific sequence differences which in
10 turn can be used to design DNA probes. A detection system using polymerase chain reaction (PCR) can then be developed. Such PCR reactions will involve combinations of oligonucleotides specific to hepatitis B virus with a vaccine-induced mutation at the amino acid residue 145
15 (Glycine to Arginine) of the major surface antigen, thereby allowing highly specific detection of these mutant viral DNAs. Briefly, viral DNA can be extracted as described in this invention. PCR reactions can be performed using specific oligonucleotides using similar cycling conditions described
20 above. Results can then be analyzed after resolving PCR products on a 1 % agarose gel.

According to known immunological procedures, it is possible to determine epitopes from protein sequences such as those in
25 Figures 4-7. Determination of these epitopes specific to hepatitis B virus with vaccine-induced mutation at amino acid residue 145 of the major surface antigen will allow the synthesis of peptides using genetic engineering methods, synthesis of the proteins, production of the antibodies,
30 development of specific diagnostic reagents, development of prophylactic and therapeutic vaccines, and antiviral agents.

A detection system for antibodies against hepatitis B virus, with a vaccine-induced mutation at amino acid residue 145 of
35 the major surface antigen, can be developed using polyvinyl microtiter plates and the sandwich method. Briefly, 50 μ l of 5 μ g/ml concentration of a hepatitis B virus (vaccine-induced mutant) peptide can be dispensed in each well of the

microtiter plates and incubated overnight at room temperature for consolidation. Similar procedures can be applied to 's' protein purified from host cells such as *Escherichia coli*. The microplate wells can be washed five times with physiological saline solution containing 0.05% Tween 20. For overcoating, 100 μ l of NaCl buffer containing 30% (v/v) of calf serum and 0.05% Tween 20 (CS buffer) can be dispensed in each well and discarded after incubation for 30 minutes at room temperature.

10

To determine antibodies specific for the vaccine-induced mutant hepatitis B virus antibodies in serum, the primary reaction can be carried out such that 50 μ l of the CS buffer and 10 μ l of a serum sample can be dispensed in each microplate well and incubated on a microplate vibrator for one hour at room temperature. After completion of the primary reaction, microplate wells are washed five times as described above.

20 In the secondary reaction, 1 ng of horseradish peroxidase labeled anti-human IgG mouse monoclonal antibodies dissolved in 50 μ l of calf serum can be dispensed in each microplate well, and incubated on a microplate vibrator for one hour at room temperature. Upon completion, wells can be washed five times in the same way. After addition of hydrogen peroxide (as substrate) and 50 μ l of O-phenyldiamine solution (as color developer) in each well, and after incubation for 30 minutes at room temperature, 50 μ l of 4M sulphuric acid solution can be dispensed in each well to stop further color development and for reading absorbance at 490nm.

35 The present invention makes possible detection of vaccine-induced mutant hepatitis B virus, in particular those carrying a mutation at amino acid residue 145 of the major surface antigen. Such mutant hepatitis B virus have hitherto escaped the detection using conventional antibody-based methods, and the present invention also provides detection systems capable of highly specific and sensitive detection at

an early stage of infection.

In addition, these features allow accurate diagnosis of patients at an early stage of the disease and also help to remove with higher efficiency blood contaminated with vaccine-induced mutant hepatitis B virus through using a screening test of donor bloods.

proteins and their antibodies under the present invention can be utilized for development of prophylactic and therapeutic vaccines, as well as, immunological pharmaceuticals. Sequence information on structural genes of these mutant viruses will be helpful in developing detection systems of the relevant protein antigens and antibodies.

Antigen-antibody complexes can be detected by known methods. Specific monoclonal and polyclonal antibodies can be raised by immunizing animals such as mice and rabbits with peptides or proteins specific to mutant vaccine-induced hepatitis B viruses. Inhibitory antiviral agents can be designed and targeted against these proteins and molecules in cell culture or in vivo.

The present invention is based on studies on an isolated virus genome with a vaccine-induced mutation at amino acid residue 145 (Glycine to Arginine) of the major surface antigen. The invention makes possible highly specific detection of these vaccine-induced mutant hepatitis B virus and provides material such as protein, polyclonal and monoclonal antibodies for development of such detection system.

We have evidence showing that when expressed in a mammalian expression system, the major surface antigen (HBsAg) of the mutant HBV reported in our invention is detected as a 22kDa protein on a Coomassie-blue stained SDS-PAGE gel, whereas the wild type HBsAg is detected as a 25kDa protein. Since the only glycosylation site on the HBsAg is located in close proximity (Asparagine at position 146) to the mutation at

position 145, it is very likely that the drastic change from Glycine to Arginine at position 145 affects the glycosylation process in the mutant HBsAg. This defective glycosylation process would in turn result in the smaller protein as
5 observed in our studies. As the antigenicity of a protein is adversely affected by the extent of its glycosylation, we predict that the mutant HBsAg reported in our invention is more antigenic than the wild type HBsAg. This prediction is further supported by the structural analysis using the method
10 developed by Hopp and Woods.

Our serological studies also indicate major differences between wild type HBV carriers and those carrying the mutant HBV reported in our invention, one such difference is that the
15 serum viral DNA load is significantly lower at around 5pg per ml as measured by an ^{125}I hybridization assay (Abbott Laboratories, U.S.A.). In marked contrast, the wild type HBV DNA load is much higher at over 100pg per ml. In addition, the anti-Hepatitis B surface antigen (anti-HBs) level is
20 detectable at 10 IU per ml in the mutant HBV reported in our invention, whereas such antibodies remained undetectable in carriers of wild type HBV.

In the process of sequence determination, we have observed
25 that a few oligonucleotides (each of them longer than 17 bases) designed according to the wild type HBV sequence, failed to hybridize to the mutant viral DNA reported in our invention. Two such oligonucleotides are located in the genome at positions 2711 to 2729 and 2902 to 2920. Our
30 results, therefore, point to the structural differences between the wild type and mutant HBV genomes.

REFERENCES

1. Oon, C-J., "Viral hepatitis B in Singapore: epidemiology, prevention and control-monitoring the hepatitis B programme and management of carriers" J. Royal College Physic. London (1997) in press.
2. Oon, C-J., "Issues associated with HBV mutant strains" J. Royal College Physic. London (1997), in press.
3. Oon, C-J., et al. "Hepatitis B surface antigen (HBsAg) mutants - their significance" Annals Acad. Med. Singapore (1997) in press.
4. Oon, C-J., "Molecular epidemiology of hepatitis B 'a' variants and mutants: significance in immune population" JAMA (1996) 12: 5-6.
5. Goh, K-T, "Hepatitis B immunization in Singapore" Lancet (1996) 348: 1385-1386.
6. Oon, C-J., et al., "Natural history of hepatitis B surface antigen mutants in children" Lancet (1996) 348: 1524-1525.
7. Harrison, T.J., "Genetic variation in hepatitis B virus", Eur. J. Gastroenter. & Hepatol. (1996) 8: 306-311.
8. Oon, C-J., et al., "Molecular epidemiology of hepatitis B virus vaccine variants in Singapore" Vaccine (1995) 13: 699-702.
9. Carman, W., et al., "Viral genetic variation: hepatitis B virus as a clinical example" Lancet (1993) 341: 349-353.
10. Harrison, T.J., "Variants of hepatitis B virus" Vox Sang (1992) 63: 161-167.

11. Harrison, T.J. et al., "Independent emergence of a vaccine-induced escape mutant of hepatitis B virus", J. Hepatol. (1991) 13: S105-107.
- 5 12. Carman, W.F. et al., "Vaccine-induced escape mutant of hepatitis B virus" Lancet (1990) 336: 325-329.